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(54) Title: RECOMBINANT MAMMALIAN CALCITONIN RECEPTORS AND USES THEREOF

(57) Abstract

A method of testing a compound to determine whether it is capable of binding to a cacitonin receptor, the method comprising a) providing a recombinant eukaryotic cell which is transfected with DNA encoding calcitonin receptor and which is capable of expressing calcitonin receptor on its surface, b) contacting the cell with the test compound, and c) detecting binding of the cell with the test compound as an indication of binding of the test compound to the receptor.

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RECOMBINANT MAMMALIAN CALCITONIN RECEPTORS AND USES THEREOF

Partial funding for the work described herein was provided by the U.S. Government, which has certain rights in the invention.

The invention relates to the field of receptors for peptide hormones such as calcitonin.

Calcitonin is a peptide hormone of 32 amino acids that was initially identified as a hypocalcemic factor secreted by the parafollicular cells of the thyroid gland in response to elevations in serum calcium (Copp et al., 1962, Endocrinology 70:638-649). The hypocalcemic effect of calcitonin is mediated primarily by direct inhibition of osteoclast-mediated bone resorption (Friedman and Raisz, 1965, Science 150:1465-1467; Warshawsky et al., 1980, J. Cell Biol. 85:682-694). Calcitonin also enhances renal calcium excretion (Haas et al., 1971, J. Clin. Invest. 50:2689-2702; Warshawsky et al., *Supra*). In addition to receptors in bone and kidney, high affinity calcitonin binding sites have been demonstrated in many different tissues including the central nervous system, testes, placenta, lung, and on spermatozoa. Cells derived from lung and breast carcinomas, as well as certain lymphoid and myeloid cell lines also express receptors for this hormone. Although the physiologic role of calcitonin in many of these tissues remains poorly understood, its action clearly extends beyond its effects on calcium homeostasis.

The unique ability of calcitonin to inhibit osteoclast-mediated bone resorption has led to its wide-spread use in the treatment of disorders of bone-remodelling, including osteoporosis, Paget's disease of bone and some forms of hypercalcemia of malignancy. In addition, calcitonin has been used in studies to treat

pancreatitis and peptic ulcer disease, and to produce centrally mediated analgesia. It has not been established whether all of the pharmacological effects of calcitonin are mediated directly by high affinity calcitonin receptors in these target tissues, or whether they are related to the cross-reaction of calcitonin with receptors for other hormones such as α or β calcitonin gene related peptide (CGRP) (Wohlwend et al., 1985, Biochem. Biophys. Res. Comm. 131:537-542), or amylin (Zhu et al., 1991, Biochem. Biophys. Res. Comm. 177:771-776), which share similarity in amino acid sequence with calcitonin. αCGRP is a product of the calcitonin gene produced by differential RNA splicing. βCGRP is a product of a separate gene but differs from αCGRP by only a single amino acid. These related ligands most likely interact primarily with their own high affinity receptors to produce hormone-specific effects, but at very high concentrations may also cross react with the receptors for the other peptides.

Summary of the Invention

The invention features a recombinant DNA which encodes a calcitonin receptor polypeptide; the recombinant DNA is preferably a cDNA encoding porcine or human calcitonin receptor polypeptide.

The recombinant DNA can be used to test a compound to determine whether it is capable of binding to a calcitonin receptor; the method involves a) providing a recombinant eukaryotic cell which is transfected with DNA encoding calcitonin receptor and which is capable of expressing calcitonin receptor on its surface; b) contacting the cell with the test compound; and c) detecting binding of the cell with the test compound as an indication of binding of the compound to the receptor.

The recombinant DNA can also be used to test a compound to determine whether it is capable of binding to a calcitonin receptor; the method involves a) mixing calcitonin receptor with labeled calcitonin and the test compound, and b) measuring the amount of label bound to the receptor as an indication of binding of the test compound to the receptor.

In preferred embodiments, the recombinant DNA encodes a receptor which is a mammalian calcitonin receptor, most preferably from a pig or a human.

In other preferred embodiments, the cell in which the recombinant DNA is expressed is a cell that does not express on its surface any other proteins capable of binding to calcitonin receptor.

In yet other preferred embodiments, the method of testing compounds further comprises the step of determining whether the test compound exhibits in its interaction with the cell a biological activity of calcitonin, most preferably this activity is an increased level of intracellular cyclic AMP, or an increased intracellular concentration of calcium.

The invention also features recombinant calcitonin receptor polypeptide expressed from the recombinant DNA, wherein the polypeptide is most preferably porcine or human calcitonin receptor polypeptide.

The invention includes a vector comprising recombinant DNA which is capable of directing the expression of a polypeptide encoded by the DNA in the vector-containing cell.

The invention also includes a method of producing a recombinant calcitonin receptor polypeptide, the method comprising, a) providing a recombinant cell transformed with DNA encoding calcitonin receptor polypeptide positioned for expression in the cell; b) culturing the transformed cell under conditions for

expressing the DNA; and c) isolating the recombinant polypeptide.

The invention also features an antibody which binds preferentially to a calcitonin receptor polypeptide, and a method of using the antibody to identify other calcitonin receptors in cells from tissues.

The invention also includes a method of identifying other calcitonin receptors in cells from tissues by screening a bacterial library expressing RNA specific for that tissue with a recombinant DNA encoding calcitonin receptor, or a portion thereof of greater than or equal 30 base pairs, which contains an identifying region unique calcitonin receptor, and detecting hybridization of the probe to the bacterial cells as an indication that the bacterial cells express RNA specific for calcitonin receptor.

Calcitonin receptor, as used herein, means any receptor in any organ or tissue to which calcitonin preferentially binds, including receptors that are related to, but not identical to, calcitonin receptor.

Recombinant DNA, as used herein, means DNA which is separated from other DNA with which it is naturally joined covalently.

A vector, as used herein, is an autonomously replicating DNA molecule.

In the methods of the invention, compounds will be tested for their ability to bind to the calcitonin receptor and for their ability to exhibit biological activity. The methods rely upon the expression of calcitonin receptor on the surface of cells that do not naturally express such proteins on their surface. The methods therefore have significant advantages over currently available methods, namely cells that naturally express calcitonin receptors, because they provide a

clean assay, free of background binding activity, and wherein cross reactivity of compounds to calcitonin-like receptor molecules is eliminated.

The invention provides for the identification of new or existing compounds that exhibit biological activity of calcitonin. While calcitonin is available for the treatment of many diseases of humans, it cannot be administered orally. Newly identified compounds that exhibit biological activity of calcitonin may be orally available.

The antibody and calcitonin receptor-specific probes of the invention can be used to locate other calcitonin receptors within tissues of a mammal, or as a diagnostic tool to identify diseased cells that express calcitonin receptors. Newly identified receptors can be used in the screening assay to identify yet other compounds that have biological activity of calcitonin that may be unique to specific tissues and organs in a mammal.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

The Drawings

The drawings are first described.

Figure 1 is a composite of graphs depicting binding of porcine calcitonin to cells. A) Porcine calcitonin binding to LLC-PK_1 cells; B) Porcine calcitonin binding to COS cells transfected with cDNA encoding porcine CTR. Insets show Scatchard analysis of the binding data.

Figure 2 is a histogram of the cAMP response in A) porcine calcitonin receptor-transfected COS cells and B)

COS cells mock-transfected with β-galactosidase. SCT = salmon calcitonin and ISO = isoproterenol.

Figure 3 is the amino acid sequence of porcine CTR aligned with the PTH-PTHrP receptor. The alignment was generated with UWGCG program GAP (Devereux et al., 1984, Nucl. Acids Res. 12:387). Shaded boxes represent identity or similarity. The bars above the sequence represent the transmembrane domains. Symbol # indicates N-linked glycosylation sites and + indicates conserved cysteines.

Figure 4A (low power) and B (high power) are emulsion autoradiographs of BIN-67 cells in culture following incubation with [^{125}I]-salmon calcitonin.

Figure 5A and B are graphs of binding of salmon calcitonin to BIN-67 cells (A), and to COS cells transfected with the human CTR cDNA (B). Insets show Scatchard analysis of binding data.

Figure 6 is a composite of graphs of binding and cAMP dose response of human and salmon calcitonins in BIN-67 cells. A) cAMP dose response curves to salmon and human calcitonin; B) Competition dissociation binding curves for [^{125}I]-human calcitonin competed with unlabeled human calcitonin; C) Human calcitonin binding to BIN-67 cells. Maximal binding was 1.92 X 10^4 cpm per sample. Insets show Scatchard analysis of binding data.

Figure 7 is a histogram of cAMP response in BIN-67 cells and in transfected COS cells. A) Mock (β-galactosidase) transfected COS cells; B) BIN-67 cells; C) COS cells transfected with human CTR cDNA.

Figure 8 is the nucleotide and predicted amino acid sequence of the human CTR cDNA clone. The first underlined nucleotide triplet represents a potential initiation codon upstream of the assigned putative transcription start site. The arrow denotes a potential cleavage site (between bp 22 and 23) for a possible

hydrophilic sequence. Four potential N-linked glycosylation sites are designated by the symbol #, and of those, the conserved sites are marked with the symbol *. Boxes mark extracellular cysteines. The seven putative hydrophilic membrane spanning domains are underlined.

Figure 9 is an autoradiogram of a northern blot hybridization experiment. Lane 1: 1 μg of LLC-PK_1 mRNA; Lane 2: 5 μg of BIN-67 cell mRNA; Lane 3: 5 μg of T-47D cell mRNA; Lane 4: 5 μg of human Giant Cell Tumor of bone mRNA. Size markers are on the left of the figure.

Cloning and Analysis of the cDNA Encoding a High Affinity Calcitonin Receptor from Porcine Cells.

The porcine calcitonin receptor was cloned by expression in COS cells, using a strategy generally described in Lin et al., 1991, Proc. Natl. Acad. Sci. USA 88:3185. A size-fractionated cDNA library was constructed from LLC-PK_1 cells (Goldring et al., 1978, Biochem. Biophys. Res. Comm. 83:434), a porcine kidney epithelial cell line that expresses approximately 3 X 10^5 calcitonin receptors per cell with an apparent dissociation constant (K_d) of approximately 3 nM (Figure 1A). Pools of mini-prep cDNA (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY) containing 10^4 recombinants were transfected into COS cells and screened for binding to radioiodinated salmon calcitonin by emulsion autoradiography (Gearing et al., 1989, EMBO 8:3667). After screening 30 pools representing 3 X 10^5 clones, two positive pools were identified from which two positive clones with cDNA inserts 2.2 and 3.9 kb in length were isolated. The 2.2 kb clone (3J8-14-FI) was a truncated

version of the 3.9 kb clone (2B5-0-I) but encoded the same open reading frame.

Expression and function of salmon CTR on transfected COS cells. Radioiodinated salmon calcitonin binds to LLC-PK_1 cells and to COS cells transfected with the cloned porcine calcitonin receptor (CTR) cDNA (Figure 1). Transfected COS cells expressed approximately 2 X 10^6 receptors per cell (assuming 10% of the cells expressed receptor) with an apparent K_d of approximately 6 nM, similar to that expressed by LLC-PK_1 cells. Bovine parathyroid hormone (1-34) [PTH(1-34)] (Juppner et al., 1991, Science 254:1024) did not compete for binding of radioiodinated salmon calcitonin to the CTR transfectants.

The cloned porcine receptor is functionally coupled to increased intracellular cAMP (Figure 2). A 4-fold increase in the concentration of intracellular cAMP was observed after incubation of porcine CTR-transfected COS cells with calcitonin, but there was no increase when cells were transfected with β-galactosidase and subsequently stimulated with calcitonin. Isoproterenol, an agonist of the β-adrenergic receptor, activated adenylate cyclase in both β-galactosidase and porcine CTR-transfected cells.

RNA analysis. Northern blot hybridization analysis of poly A^+ mRNA from LLC-PK_1 cells and pig organs demonstrated a single transcript of approximately 4.2 kb. Expression of this mRNA was most abundant in the brain but was also present in other tissues.

Analysis of the deduced amino acid sequence of the porcine CTR. Analysis of the deduced amino acid sequence of the porcine CTR (Figure 3) revealed a molecule with an usual structure. Searches of nucleic acid and protein

sequence databases have not identified sequences similar to porcine CTR. A Kyte-Doolittle hydropathy analysis (Kyte and Doolittle, 1982, J. Mol. Biol. 157:105) indicated seven or eight regions of hydrophobic amino acid sequences that could generate transmembrane domains. The porcine CTR has no significant sequence identity (12%) to any of the approximately 120 cloned receptors that are thought to span the membrane seven times and to interact with G proteins (Attwood et al., 1991, Gene 98:153).

The NH_2-terminal hydrophobic domain, a putative hydrophobic signal sequence (Von Heijne, 1986, Nucl. Acids Res. 14:4683), precedes a long NH_2-terminal domain (147 amino acids with 3 potential N-linked glycosylation sites) that is presumed to be extracellular. There is a short cytosolic loop between helices V and VI that is not similar to corresponding regions of other adenylate cyclase-coupled receptors; this region is thought to couple to $G_{s\alpha}$. This unusual structural feature of the porcine CTR could account for the observed coupling of the receptor to different G proteins in cultured osteoclasts (Zadi et al., 1990, J. Endocrinol. 126:473) and the coupling that is observed during different phases of the cell cycle in LLC-PK_1 cells (Chakraborty et al., 1991, Science 251:1078). There is a striking degree of amino acid sequence similarity between the porcine CTR and the PTH-PTH related peptide (PTH-PTHrP) receptor, which is also different from other G-protein coupled receptors (Figure 3; Juppner et al., 1991, Science 254:1024). Although the PTH-PTHrP receptor is more than 100 amino acids longer than the porcine CTR, there is an overall approximate 32% identity and an approximate 56% similarity between the sequences of the two receptors. A stretch of 17 out of 18 amino acids around the putative transmembrane domain VII are identical. Also, two out of

four N-linked glycosylation sites and the position of seven out of eight potentially extracellular cysteines are conserved (Figure 3). Major differences between the two receptors appear to lie in their N-terminal and carboxy-terminal domains, where gaps exist in the porcine CTR sequence relative to the PTH-PTHrP sequence. Both receptors also activate adenylate cyclase (Figure 2; Juppner et al., 1991, Science 254:1024). The structural similarity of the porcine CTR and the PTH-PTHrP receptor suggests that they represent members of a new class of seven transmembrane-spanning G protein-coupled receptors that activate adenylate cyclase.

Cloning and Analysis of the cDNA Encoding a High Affinity Calcitonin Receptor from Human Cells.

A size-fractionated library with inserts greater than 2 kb in length consisting of 17 million recombinants was constructed from a rare small cell human ovarian carcinoma cell line, BIN-67, previously reported to respond to calcitonin with increases in cAMP (Upchurch et al., 1986, J. Bone and Mineral Res. 1:299). Poly A^+ mRNA was prepared from cells by the proteinase K/SDS method (Sambrook et al., 1989, Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY) and purified by chromatography through oligo-dT cellulose (Collaborative Research, Bedford MA). The mRNA was converted to double stranded cDNA (Gubler and Hoffman, 1983, Gene 25:263) and after separation on a potassium acetate gradient (20%/5%), size-fractionated cDNA greater than 2 kb was ligated into the eukaryotic expression vector pcDNA-1 (Invitrogen, San Diego, CA). An aliquot of the ligated plasmid-cDNA library was electroporated into MC1061/P3 E. coli with a Bio-Rad Gene Pulser (Richmond, CA), using pulse conditions as follows: 200 ω,

2.5 kV, and 2.5 μF) in 0.2 cm gap cuvettes. The bacteria were then diluted and plated on 15 cm petri dishes containing selective agar. Two nylon filters (ICN, Cleveland, OH) were sequentially imprinted with plasmid-containing clones by placing them in contact with the bacterial colonies on the agar. The imprinted filters were screened by in situ hybridization as follows: The colonies were lysed by placing the filters face up on filter paper (Whatman No. 1) that was soaked with a solution containing 5% SDS in 2 X standard saline citrate (SSC). Released DNA from the bacteria was denatured by heating at 650 watts for 2.5 minutes in a microwave oven. Filters were then washed in 5 X SSC + 0.1% SDS, followed by 5 X SSC without SDS, and then transferred to microwave cooking bags (Kapak, Minneapolis, MN). Filters were prehybridized in 5 X SSC; 40% formamide; 50 mM sodium phosphate; 5 X Denhardts solution; sheared, denatured salmon sperm DNA (0.5 mg/ml) and 0.2% SDS. A radiolabeled cDNA probe was prepared from a 1100 base pair (bp) restriction fragment of the porcine CTR open reading frame as described above, using the Klenow fragment of prokaryotic DNA polymerase I (Pharmacia, Uppsala, Sweden), in the presence of random sequence hexanucleotides and [α^{32}P] dCTP (New England Nuclear/Dupont, Boston, MA). Hybridization was carried out in a solution containing 5 X SSC; 40% formamide, 20 mM sodium phosphate; 5 X Denhardts solution; 0.1 mg/ml salmon sperm DNA and the radioactive probe for 12-24 hours. Following hybridization, filters were washed in 2 X SSC + 0.2% SDS at room temperature for 30 minutes followed by a second wash in 0.5 X SSC + 0.2% SDS at room temperature for 4 hours with multiple buffer changes. Autoradiography was performed by exposing the filters to Kodak XAR-5 film (Rochester, NY) for 12-72 hours, with an intensifying screen. Colonies that hybridized with the

labeled probe on both filters from a matching pair were isolated from the original agar plate, grown overnight, and the plasmid DNA they contained was isolated using an alkaline lysis procedure (Sambrook et al. *Supra*).

Approximately 55,000 colonies were transferred to nylon filters and screened to yield one positive clone which contained an insert of 3,605 bp. Positive clones were retested by Southern blot hybridization. A human cDNA clone, named HCTR-BIN67, which hybridized to the porcine cDNA restriction fragment probe was chosen for further study.

Sequencing of the Human CTR cDNA. Restriction fragments of a CTR-containing cDNA clone were subcloned into M13 phage vectors mp18 and mp19 (Boehringer-Mannheim, Indianapolis, IN) for sequencing. Both strands were sequenced by the dideoxynucleotide chain termination procedure with modified T7 polymerase (United States Biochemical Corp., Cleveland, OH). The cloned plasmid containing the CTR cDNA was also used in some reactions for sequencing template DNA ("double-stranded" DNA sequencing). Complimentary oligonucleotides to the sequenced DNA were synthesized for use as sequencing primers using an oligonucleotide synthesizer.

Transfection of COS-M6 (COS-7 subclone) with human CTR cDNA. Plasmid DNA was used to transfect COS-M6 cells growing in 10 cm petri dishes (Falcon, Lincoln Park, NJ) using the DEAE-dextran/chloroquine procedure (Seed and Aruffo, 1987, Proc. Natl. Acad. Sci. USA 84:3365). Forty-eight hours after transfection, cells were incubated in the presence of either iodinated calcitonin for ligand binding studies, or unlabeled calcitonin for the assay of intracellular cAMP.

Binding of radiolabeled salmon and human calcitonin to cultured cells. Human small cell ovarian cell carcinoma cells (BIN-67) were grown in 10 cm petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (vol/vol) heat inactivated fetal calf serum (GIBCO) and enriched with 25% Ham,s F12 medium (GIBCO). Cells at near confluency, were passed approximately twice a week by trypsinization using 0.25% trypsin (GIBCO). Radioligand binding assays were performed in triplicate as follows. The cells were washed, trypsinized and counted in an automated cell counter (Coulter, Hialeah, FL). They were distributed into 12 X 75 mm glass tubes at 5 X 10^5 cells per tube in a volume of 200 μl of binding buffer [phosphate buffered saline (PBS), pH 7.4, 11 mM glucose, 0.5% bovine serum albumin] plus 200 pM of either [^{125}I]-salmon calcitonin (Peninsula Laboratory, Belmont, CA), or [^{125}I]-human calcitonin (Amersham, Arlington Heights, IL) in the presence of appropriate amounts of unlabeled ligand (Sigma, St. Louis, MO). The mixture was incubated for 14-16 hours at 4°C. The cells were washed by layering 100 μl of cell suspension over 400 μl of 10% sucrose (wt/vol) in a mini-microfuge tube (Bio-Rad) and centrifuging at maximum speed in a microfuge for three minutes to pellet the cells. The supernatant was removed by aspiration, and the portion of the tube containing the cell pellet was cut off and assayed for radioactive content in a gamma counter (TM Analytic, Elk Grove Village, IL). Ligand binding to COS-M6 cells transfected with a human CTR-containing plasmid was performed using the same technique.

cAMP assay. BIN-67 cells or COS-M6 cells were grown in 10 cm petri dishes. Forty eight hours before the cAMP assay, the COS-M6 cells were transfected with either human CTR cDNA, or with β-galactosidase cDNA which served

as a control. After 24 hours, the transfected COS-M6 cells and the BIN-67 cells were trypsinized and transferred to plastic trays containing 24 16 mm wells (Falcon) at an initial plating density of 5 X 10^4 cells/well. To test for hormone-induced cAMP responses, the medium was removed from each well and the cells were washed with PBS supplemented with calcium and magnesium. Triplicate groups of cells were incubated for 20 minutes at 37°C at room temperature with either test buffer [PBS supplemented with calcium, magnesium, 0.25% bovine serum albumin, 11 mM glucose and 1 mM 3-isobutyl-methyl-xanthine (IBMX)], or with 4 mM isoproterenol or peptide hormone at the appropriate concentrations. Reactions were stopped by placing the culture plates in a water bath at 100°C until all liquid had evaporated. The plates were stored at -20°C until assayed. The cAMP assay was performed by adding 1.0 ml of 50 mM sodium acetate buffer, pH 6.2, to each well. Dried cells were scraped into this buffer, transferred to glass tubes and centrifuged at 500 X g for 10 minutes. Aliquots of supernatant were assayed for cAMP using a radioimmunoassay kit (New England Nuclear/Dupont cAMP [^{125}I] Radioimmunoassay Kit, Dupont).

<u>Emulsion autoradiography of BIN-67 cells.</u> Cells were grown on glass chamber slides (Nunc, Kamstrup, DK). The medium was removed by aspiration and the cells were incubated in binding buffer (PBS supplemented with 11 mM glucose, 0.5% bovine serum albumin) with [^{125}I]-salmon calcitonin (200 pM) with or without 10^{-6} M unlabelled salmon calcitonin (Sigma). After 5 washes in ice cold PBS supplemented with calcium and magnesium, the cells were fixed in PBS plus 2% formaldehyde, coated with Kodak NTB2 emulsion and exposed for 1-3 weeks at 4°C, after which they were developed and counterstained with Giemsa.

Northern blot hybridization analysis. Samples containing 5 μg of poly A^+ RNA prepared from BIN-67 cells, T-47D cells (a human breast carcinoma cell line which expresses well characterized calcitonin receptors), and human giant cell tumor of bone tissue (hGCTu), and 1 μg of poly A^+ RNA prepared from LLC-PK_1 cells were electrophoresed on a 1% agarose gel containing formaldehyde and transferred by capillary action, using 10 X SSC, to a supported nitrocellulose filter (Schleicher and Schuell). The filter was heated for 90 minutes at 80°C under vacuum. Prehybridization was performed for 12-16 hours in 40% formamide (vol/vol); 5 X SSC; 50 mM sodium phosphate, pH 7.2; 0.5 mg salmon sperm DNA per ml; 5 X Denhardt's solution and 0.2% SDS. Hybridization was performed at 42°C for 12-16 hours using a probe consisting of a human CTR cDNA Sac I digested restriction fragment of approximately 950 bp, labeled with [α^{32}P]dCTP (New England Nuclear/Dupont) by random primer labeling. The hybridization solution contained 40% formamide; 5 X SSC; 50 mM sodium phosphate; 5 X Denhardt's solution and 0.1 mg/ml salmon sperm DNA. The filters were washed two times with 2 X SSC, 0.2% SDS for 15 minutes at room temperature followed by four 20 minute washes in 0.2 X SSC, 0.2% SDS at 60°C. Hybridized RNA was visualized following exposure of the filters to Kodak XAR-5 film for 24-72 hours at -70°C, with intensifying screen.

Characterization of Human CTRs in the BIN-67 cell line using [^{125}I]-salmon calcitonin emulsion autoradiography. The BIN-67 cell line was isolated from a trypsin digest of a human metastatic pelvic nodule derived from a primary ovarian small cell carcinoma, a rare tumor composed of poorly differentiated cells of uncertain developmental origin (Upchurch et al., 1986, J. Bone and Mineral Res. 1:299; Dickersin et al., 1982, Cancer

49:188; Moll et al., 1983, Lab. Invest.49:599). The cultured cell line preserves the mixed character of the primary tumor with both large and small cell components. The small cells contain small dark nuclei with scanty cytoplasm which often grow in mounds on tissue culture plastic. Adjacent areas reveal larger cells with dense abundant cytoplasm and nuclei with prominent nucleoli. These cells grow with cytoplasmic extensions which spread out over the surface of the culture dish but do not tend to adhere closely to adjacent cells. Figure 4A and 4B are two emulsion autoradiographs prepared from cells after incubation with [^{125}I]-salmon calcitonin. The presence of receptors for salmon calcitonin are indicated by the dense silver granules conforming to the outline of individual cells. In Figure 4A it can be seen that the cells are clearly heterogeneous with respect to the expression of calcitonin receptors. Figure 4B is a high power view of a cell which expresses abundant calcitonin receptors. The specificity of salmon calcitonin for these cells was demonstrated by the fact that incubation with excess unlabelled salmon calcitonin competed out all of the label.

<u>Characterization of radioiodinated calcitonin binding in BIN-67 cells.</u> Scatchard analysis of binding data in BIN-67 cells using radiolabeled salmon calcitonin was consistent with a single class of high affinity calcitonin binding sites with a calculated K_d of 0.42 nM (Figure 5A). The average number of specific binding sites per cell was 143,000. Scatchard analysis of binding studies on BIN-67 cells using radiolabeled human calcitonin demonstrated a 10-fold lower affinity of these cells for human calcitonin with a K_d of approximately 4.6 nM (Figure 6C).

Analysis of data from competition dissociation studies following incubation of BIN-67 cells with [^{125}I]-human calcitonin in the presence of increasing concentrations of unlabeled salmon calcitonin revealed an apparent 50% inhibitory concentration (IC_{50}) in the range of 0.6-0.7 nM (Figure 6B). Parallel studies using [^{125}I]-human calcitonin with increasing concentrations of unlabeled human calcitonin confirmed a 5-10-fold lower affinity (IC_{50} approximately 3-7 nM) of BIN-67 cells for human calcitonin compared to salmon calcitonin (Figure 6B). In addition, the peptide hormones, secretin and PTH, failed to displace radiolabeled salmon or human calcitonin binding even at concentrations as high as 10^{-5} M. Additional studies confirmed that calcitonin binding sites were saturable with maximal binding at $4^{0}C$ occurring by approximately 12 hours.

<u>Characterization of radioiodinated calcitonin binding to COS-M6 cells transfected with the human CTR cDNA.</u> COS-M6 cells, which do not express CTR or CTR-like molecules on their cell surface, were transfected with the plasmid HCTR-BIN67 containing the human CTR cDNA and incubated with either radioiodinated salmon or human calcitonin. Scatchard analysis of binding data was consistent with the presence of a single class of high affinity calcitonin binding sites (Figure 5). Assuming 10% transfection efficiency (based on previous studies), the number of receptors per cell was approximately 1.4 X 10^{6}. The apparent K_d for salmon calcitonin was 0.44 nM, which agrees closely with the apparent K_d for this peptide in native BIN-67 cells (0.42 nM). As in the native cells, the expressed human CTR in COS-M6 cells had an approximate 10-fold lower affinity for human calcitonin (6.4 nM) compared to salmon calcitonin. The specificity of binding in COS cells transfected with the human CTR

cDNA was indicated by the failure of other hormones, including PTH or secretin, to compete for binding with either radioiodinated salmon or human calcitonin.

Characterization of hormone-induced cAMP responses in BIN-67 cells and in COS-M6 cells transfected with the human CTR cDNA. As shown in Figure 7A, BIN-67 cells exhibited a dose-dependent increase in cAMP levels in response to salmon or human calcitonin. The 50% maximal effective concentrations (EC_{50}) for salmon calcitonin (approximately 0.7 nM) and human calcitonin (approximately 3.0 nM) illustrate the greater sensitivity of these cells to salmon calcitonin. These data are consistent with the dissociation constants for these peptides based on binding studies with radiolabeled ligands.

To determine whether the human CTR cDNA encoded a calcitonin binding protein that can couple to adenylate cyclase, COS-M6 cells were transfected with the human CTR cDNA and then incubated with calcitonin for 20 minutes in the presence of the phosphodiesterase inhibitor, IBMX. As shown in Figure 7C, transfected cells had approximately a 4-fold increase in cAMP levels when incubated with maximal stimulatory concentrations of salmon calcitonin. The range was 2 to 4-fold in multiple experiments. This increase is considerably lower than the magnitude of the response in BIN-67 cells (approximately 9 to 24-fold, Figure 7B). COS-M6 cells transfected with a β-galactosidase cDNA failed to increase cAMP levels above control levels following incubation with salmon calcitonin. The specificity of the salmon calcitonin-induced cAMP response was further demonstrated by the failure of PTH to induce a response (Figure 7C), and by the fact that incubation with secretin at concentrations up to 10^{-6} M also failed to

induce a response. Finally, isoproterenol, an agonist of the β-adrenergic receptor, increased cAMP levels in both the mock-transfected and the human CTR-transfected COS cells.

Analysis of the human CTR cDNA predicted amino acid sequence. Sequence analysis of the 3,605 bp human CTR cDNA (Figure 8) revealed an open reading frame beginning at bp 250, which encodes a putative peptide of 490 amino acids. Comparison of this deduced amino acid sequence to that of the putative porcine CTR demonstrates a sequence identity of 73% and a similarity of 89%. The putative human CTR is eight amino acids longer than the peptide deduced from the porcine cDNA. The human CTR contains a second in frame AUG located at bp 195, or 55 bp upstream from the assigned start site. Both AUG codons have an A at the -3 position consistent with and sufficient for a consensus start site, although neither fits the strict consensus CC (A,G) CC AUG G (Kozak, 1984, Nucl. Acids Res. 12:857). An N-terminal domain encoded by the upstream AUG would contain mostly polar, hydrophilic residues and does not conform to the general outline of a signal peptide (von Heijne, 1986, Nucl. Acids Res. 14:4683). The downstream AUG, on the other hand, encodes a putative signal-like peptide, including a central hydrophobic domain (the h-region) flanked by polar regions consistent with the general outline of a signal peptide. The most likely cleavage site for this putative signal peptide falls between residues 22 and 23 (von Heijne, 1986, Nucl. Acids Res. 14:4683). The assignment of the human CTR cDNA start site to the downstream AUG at bp 250 is strongly supported by the positive alignment of identical and similar amino acid sequences, including a homologous N-terminal hydrophobic sequence encoded by the open reading frame of the porcine CTR cDNA (Figure 1).

The porcine CTR cDNA does not contain an analogous upstream start site to that of the human CTR cDNA, but instead possesses an in frame stop at 27 bp upstream from its start site.

When compared to other G protein-coupled hormone receptors, the deduced amino acid sequence of the human CTR shares many of the unusual structural features exhibited by the porcine CTR. A hydropathy plot (Kyte and Dolittle, 1982, J. Mol. Biol. 157:105) of the human CTR exhibits seven hydrophobic regions flanked by several charged residues which could form α-helical membrane spanning domains. The 22 residue putative signal sequence precedes a 124 amino acid presumed exoplasmic domain which includes three potential N-linked glycosylation sites that are conserved in the receptor from the two species. Both the human and porcine CTRs contain an usual hydrophobic sequence near the carboxy-terminus consisting of a series of amino acids containing alanine as the predominant residue. This sequence is considerably shorter in the human CTR (amino acids 442-451) compared to the porcine sequence (amino acids 423-439) and is therefore not long enough to form a membrane spanning domain. Both CTRs possess an unusually short cytosolic loop between helices V and VI. In other G-protein coupled receptors, this region is thought to couple to $G_{s\alpha}$.

A major area of divergence between the human and porcine calcitonin receptors falls between the first and second transmembrane hydrophobic domains where the human CTR contains an inserted sequence of 16 consecutive amino acids not found in the porcine sequence (amino acids 176-191). This insert provides the human CTR with a longer intracellular loop between the first and second predicted transmembrane helices.

Searches of nucleic acid databases (Genbank and EMBL) and protein sequence databases (Genbank Translated Databases, PIR, and Swiss-Prot) identified the rat secretin receptor as the only published sequence which exhibits significant similarity to the human (or porcine) CTR. The recently cloned receptor for PTH-PTHrP (opossum kidney) is also similar to the human and porcine CTR (Juppner et al., 1991, Science 254:1024). A statistical analysis was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service (Altschul et al., 1990, J. Mol. Biol. 215:403) to compare the human CTR to the sequences in the database, including the approximately 120 receptor proteins with seven putative transmembrane domains thought to couple G-proteins. Sequence identity to members of this database, excluding the receptors for secretin and PTH, was less than 21%, with a Highest Scoring Hit Extension of 73 histogram units, compared to 163 units for secretin. The percent identity of the human CTR with either the PTH-PTHrP or secretin receptors is 34% with 58% similarity. The secretin receptor is 30% identical and 54% similar to the human CTR. The PTH and secretin receptors are even more closely related to each other with approximately 44% identity.

All of the related receptors human CTR, porcine CTR, PTH, PTH-PTHrP and secretin, possess homologous signal peptide-like N-terminal domains. The six cysteines in the human and porcine CTRs distal to the putative signal peptide site and proximal to the first membrane spanning domain are conserved without any gap required for their alignment. In the secretin receptor only four of the cysteine residues are conserved, diverging also at the cysteine residue just proximal to the first membrane spanning domain. In addition, two other extracellular cysteine residues are conserved at

sites in the putative second and third extracellular domains of all three receptor types. Of the four potential N-linked glycosylation sites in the human N-terminal extracytoplasmic domain, three are conserved in the human and porcine CTRs; the distal two sites are also conserved in the CTRs and the PTH receptors. The secretin receptor preserves one N-linked glycosylation site nearest the first transmembrane domain, which is in a nearly identical position in the PTH and calcitonin receptors. This glycosylation site is displaced by only one amino acid toward the N-terminus relative to the first transmembrane domain in the secretin receptor compared to the PTH-PTHrP and CTR receptors.

The major areas of divergence in these receptors occurs in both the extracellular and cytoplasmic regions where gaps exist in the CTR and secretin receptor sequences relative to the longer PTH-PTHrP sequence. Nevertheless, some areas of sequence similarity and identity also exist in the C-terminal domains of these receptors all of which are known to be functionally coupled to adenylate cyclase (Murad et al., 1970, Proc. Natl. Acad. Sci. USA 65:446; Juppner et al., 1991 Science 254:1024; Ishihara et al., 1991, EMBO J. 10:1635).

RNA analysis. Northern blot hybridization analysis using a human CTR cDNA probe was performed on RNA from BIN-67 cells and T-47D cells, and on RNA prepared from hGCTu cells. The hGCTu cells possess large numbers of multinucleated giant cells which express phenotypic features of osteoclasts, including the presence of calcitonin receptors (Goldring et al., 1987, J. Clin. Invest. 79:483). RNA from the porcine LLC-PK_1 cells was included as a reference. A single transcript of approximately 4.2 kb was evident in all of the samples (Figure 9). The analysis was performed on the same blot

under moderately stringent wash conditions (60°C in 0.2 X SSC). The extremely high levels of CTR mRNA in the LLC-PK_1 cells (they express approximately 3 X 10^5 CTRs per cell) were evident from the moderately labeled band seen in Figure 9, when only one fifth of the mRNA was used compared to that used for the human cells despite the use of stringency conditions which were not optimized for species cross hybridization. Of the three human samples, BIN-67 and hGCTu cells contained much higher levels of CTR-specific mRNA than did T-47D cells.

Methods for Testing Compounds for Binding to the Human Calcitonin Receptor.

In the methods of the invention, compounds will be tested for their ability to bind to the calcitonin receptor, and for their ability to exhibit a biological activity of calcitonin. Calcitonin is currently used as a therapeutic agent to treat diseases characterized by abnormal bone-remodelling, including osteoporosis, Paget's disease of bone, and some forms of hypercalcemia associated with malignancy. A major disadvantage of calcitonin as a therapeutic agent is it's lack of oral availability. The invention provides methods for identification of compounds that bind to the calcitonin receptor and that exhibit biological activity of calcitonin. Large numbers of compounds can be tested using the methods of the invention. New or existing compounds that exhibit significant calcitonin activity in the assay may be orally available and treatment of humans with such compounds may therefore provide significant advantages to the patient over treatment with calcitonin.

Screening of compounds with potential CTR binding activity can be accomplished in a competition assay by incubating the receptor with labeled calcitonin and the compound to be tested under the standard binding

conditions described above. If the compound binds to the CTR, calcitonin will be displaced from the CTR if already bound, or will be inhibited from binding to the CTR if not already bound. In either case, at the end of the incubation period, the amount of label associated with the receptor is an indication of the amount of calcitonin bound to the receptor and therefore an indication of the ability of the test compound to compete with calcitonin for binding.

With regard to the components of the assay, human and salmon calcitonin are available commercially and can be labeled with ^{125}I or another suitable label such as biotin. The test compound is any newly synthesized compound or any available compound off the shelf. The receptor can be expressed on cell membranes, for example COS-M6 cells transfected with the cDNA encoding either porcine or human CTR. These cells can be transiently transfected with cDNA encoding CTR as described above, or can be stably transfected as follows: Cells can be cotransfected with the plasmid 3J8-14-F1 or HCTR-BIN67 and plasmid containing a selectable marker such as the neomycin resistance gene. Alternatively, 3J8-14-F1 or HCTR-BIN67 and a plasmid encoding neomycin resistance can be combined and the resulting construct can be transfected into cells. In either case, transfected cells are incubated in medium containing G418, such that only cells that are stably transformed to neomycin resistance will survive. These cells will also contain DNA sequences specifying human CTR. Cells that stably express the human CTR can be identified by the methods described above. This technology is common in the art and can be found in the Molecular Cloning Manual (Sambrook et al., *Supra*).

The invention is not limited to the use of COS-M6 cells in that any other cell line that does not express

proteins that bind to calcitonin can also be used in the methods of the invention. The invention is also not limited to plasmids encoding porcine or human CTR; rather, any plasmid encoding a calcitonin receptor can be used.

The receptor can also be used in soluble cell-free form as described below. The cDNA encoding porcine or human CTR can be expressed under the control of inducible promoter/enhancer sequences that when activated, drive the expression of high levels of CTR following transfection of the construct into the appropriate cells. Methods of inducing high levels of expression of a protein in cells are common in the art and can be found for example in the Molecular Cloning Manual (Sambrook et al. *Supra*). Receptor molecules can be purified from the transfected cells using commonly available biochemical techniques, including affinity chromatography using a column containing bound calcitonin analog(s) which can be coupled to a matrix without loss of binding activity.

Alternatively, the cDNA encoding porcine or human CTR can be cloned into a baculovirus expression system, using technology that is standard in the art (e.g., Summers and Smith, 1987, A Manual of Methods for Baculovirus Vectors and Insect Cell Procedures. Texas Agricultural Experiment Station, Bulletin 1555, Texas A&M University, College Station, TX). Receptors expressed in this way can be purified as described above.

Following incubation of the components in the standard binding assay, unbound receptor or receptor that has bound to it calcitonin or the test compound can be isolated from unbound components by taking advantage of its differential solubility in the presence of polyethylene glycol. High molecular weight receptor molecules are selectively precipitated in a solution of polyethylene glycol. For example, an identical method

has been successfully used to purify the insulin receptor (Marshall et al., 1985, J. Biol. Chem. 260:4128). The precipitate can be removed from unbound material by centrifugation and the amount of radioactivity in the precipitate can be measured in a gamma counter.

The methods described above therefore provide a useful screening procedure for the identification of compounds that bind calcitonin receptor. To identify compounds that also exhibit biological activity of calcitonin, COS-M6 cells, or any other cell line that does not express proteins on their surface that bind to calcitonin, are transfected with the cDNA encoding CTR. Test compounds or human or salmon calcitonin which serve as a controls, can be added to these cells and the mixture is incubated under the standard binding assay conditions as described above. Following incubation, cells are harvested and the levels of intracellular cAMP will be measured as described. An increase in the intracellular cAMP content in cells treated with the test compound that is similar to the increase in cAMP levels when the cells are treated with calcitonin, is an indication that the test compound exhibits a biological activity of calcitonin.

A second test for biological activity involves the use of the calcium sensitive dye fura-2-acetoxymethyl ester (Molecular Probes). This dye alters its fluorescent pattern when bound to calcium. When cells are treated with calcitonin, they exhibit an increase in calcium content. Thus, cells incubated in the presence of both calcitonin and the dye will have a different fluorescent pattern than cells that are not treated with calcitonin. If a test compound exhibits this biological activity of calcitonin when added to COS-M6 cells transfected with a cDNA encoding CTR in the presence of fura-2-acetoxymethyl ester, these cells should also

exhibit altered fluorescence compared to untreated cells.

Antibodies and Probes Specific for the Calcitonin Receptor. The invention also features antibodies and probes specific for CTR. Such antibodies or probes can be used for a variety of purposes including the location of other CTRs within tissues in a mammal which may provide insight into additional functions of calcitonin in mammals, and more importantly as a diagnostic tool, wherein cells that are associated with disease and that express CTRs on their surface can be identified.

Antibodies specific for the CTR can be generated in several ways. The procedures described below use as an example, the human CTR, but are not limited solely to the use of human CTR.

1) COS-M6 cells transfected with cDNA encoding human CTR as described above, can be used to immunize a rabbit or other mammal. These cells express human CTR on their surface but do not express other proteins with structural similarity to human CTR. Serum from inoculated rabbits can be obtained periodically and polyclonal antibody to human CTR contained therein can be purified using common techniques available in the art such as those described in Sambrook et al. (*Supra*).

2) Another method useful for the generation of antibodies involves cloning the cDNA encoding human CTR into a bacterial expression vector such that the CTR sequences are in frame with a bacterial gene, for example β-galactosidase. Bacteria that are transformed with such a construct will produce a fusion protein comprising human CTR and β-galactosidase. The fusion protein can be used to immunize a rabbit or other mammal that will then synthesize antibody specific for both proteins. This technology is also common in the art and is taught in Sambrook et al. (*Supra*).

3) Antibodies can also be generated in a rabbit or other mammal using as antigens peptides that are synthesized in a peptide synthesizer. The amino acid sequence of these peptides is identical to the amino acid sequence of the CTR deduced from the cDNA sequence described above. Such technology is also common in the art and is described in Sambrook et al. (*Supra*).

4) Human CTR that is purified according to the methods described above can also be used as an antigen for the generation of antibodies in a rabbit or other mammal.

The antibody can be used as a diagnostic tool to locate diseased cells that express calcitonin receptor using any of the methods for such purposes that are available in the art. For example, immunofluorescent or radioactive labeling techniques can be performed on tissues or individual cells as a means to identify cells, or to sort cells that express calcitonin receptor.

The antibody can also be used to screen bacterial expression libraries for the presence of calcitonin receptor, or for molecules that are related to the calcitonin receptor for example, CGRP or amylin receptor. Bacterial expression libraries specific for individual tissues are available, or can be made using the standard technology described in Sambrook et al. (*Supra*). Methods for screening such libraries using an antibody are also described in Sambrook et al (*Supra*).

In a manner similar to that described above, a probe specific for calcitonin receptor can be used as a diagnostic tool or to screen bacterial expression libraries specific for tissues for the expression of a calcitonin receptor. This can be accomplished using the methods described above or using any other conventional techniques e.g., those described in Sambrook et al. (*Supra*). The probe is the calcitonin receptor-encoding

sequence or a calcitonin receptor-specific probe that contains at least 30 base pairs that are unique to the calcitonin receptor gene. Such sequences can be easily identified using a sequence data base and a computer.

DNA from bacteria that express calcitonin receptor molecules can be isolated using the methods described in the invention. This DNA can be tested in the transfection assays described above for the expression of receptors that bind calcitonin. Expressed receptors can also be tested in the cAMP assay described above to determine whether they are coupled to adenylate cyclase.

The identification and subsequent isolation of DNA encoding tissue-specific calcitonin receptors is an important aspect of the invention, because it allows for the screening of compounds that specifically bind to these receptors. It is likely that not all calcitonin receptors in a mammal are identical in their structure and in their biological function. Furthermore, it is important to identify and characterize calcitonin receptor-related molecules in individual tissues because these molecules may play important roles in the biological function of calcitonin or related peptides. Receptors so identified can also be used in the assay described above for the identification of yet other compounds that bind to such receptors because compounds that are effective in one tissue may not be equally effective in another tissue.

Deposit

The plasmid HCTR-BIN67, has been deposited with the American Type Culture Collection on November 14, 1991, and bears the accession number ATCC No. 75144. Applicants acknowledge their responsibility to replace should the plasmid loose viability before the end of the

term of a patent issued hereon, and their responsibility to notify the American Type Culture Collection of the issuance of such a patent, at which time the deposit will be made available to the public. Prior to that time the deposit will be made available to the Commissioner of Patents under the terms of CFR §1.14 and 35 USC §112.

Claims

1. A method of testing a compound to determine whether it is capable of binding to a calcitonin receptor, said method comprising:

a) providing a recombinant eukaryotic cell which is transfected with DNA encoding calcitonin receptor and which is capable of expressing calcitonin receptor on its surface,

b) contacting said cell with said test compound, and

c) detecting binding of said cell with said test compound as an indication of binding of said compound to said receptor.

2. A method of testing a compound to determine whether it is capable of binding to a calcitonin receptor, said method comprising:

a) mixing calcitonin receptor with labeled calcitonin and the test compound, and

b) measuring the amount of label bound to said receptor as an indication of binding of said test compound to said receptor.

3. The method of claim 1 or 2, wherein said receptor is a mammalian calcitonin receptor.

4. The method of claim 1 or 2, wherein said receptor is porcine calcitonin receptor.

5. The method of claim 1 or 2, wherein said receptor is human calcitonin receptor.

6. The method of claim 1, wherein said cell does not express on its surface any other proteins capable of binding to calcitonin receptor.

7. The method of claim 1, further comprising the step of determining whether said test compound exhibits in its interaction with said cell a biological activity of calcitonin.

8. The method of claim 7, wherein said biological activity is an increased level of intracellular cyclic AMP.

9. The method of claim 7, wherein said biological activity is an increase in intracellular calcium.

10. The method of claim 2, wherein said label is ^{125}I.

11. Recombinant calcitonin receptor polypeptide.

12. The polypeptide of claim 11, wherein said polypeptide is mammalian calcitonin receptor.

13. The polypeptide of claim 12, wherein said polypeptide is porcine calcitonin receptor.

14. The polypeptide of claim 12, wherein said polypeptide is human calcitonin receptor.

15. The polypeptide of claim 13, comprising an amino acid sequence substantially identical to the amino acid sequence shown in Figure 3.

16. The polypeptide of claim 14, comprising an amino acid sequence substantially identical to the amino acid sequence shown in Figure 8.

17. Recombinant DNA which encodes the polypeptide of claim 11, 12, 13, 14, 15 or 16.

18. The recombinant DNA of claim 17, wherein said DNA is cDNA.

19. The recombinant DNA of claim 17, wherein said DNA encodes a mammalian calcitonin receptor.

20. The recombinant DNA of claim 19, wherein said DNA encodes a porcine calcitonin receptor.

21. The recombinant DNA of claim 19, wherein said DNA encodes a human calcitonin receptor.

22. The recombinant DNA of claim 20, wherein said DNA is included in the plasmid 3J8-14-F1.

23. The recombinant DNA of claim 21, wherein said DNA is included in the plasmid HCTR-BIN67.

24. A vector comprising the recombinant DNA of claim 17, said vector being capable of directing the expression of the polypeptide encoded by said DNA in a vector-containing cell.

25. A cell which contains the recombinant DNA of claim 17.

26. The cell of claim 25, said cell being a eukaryotic cell.

27. The cell of claim 26, said cell being a mammalian cell.

28. The cell of claim 27, said cell being a COS cell.

29. A method of producing a recombinant calcitonin receptor polypeptide, said method comprising,

a) providing a cell transformed with DNA encoding calcitonin receptor polypeptide positioned for expression in said cell;

b) culturing said transformed cell under conditions for expressing said DNA; and

c) isolating said recombinant calcitonin polypeptide.

30. An antibody which binds preferentially to calcitonin receptor.

31. A method of identifying a calcitonin receptor in a cell from a tissue, said method comprising

a) growing bacterial cells that express protein specific for said tissue on a solid support;

b) binding to said bacteria an antibody specific for the calcitonin receptor or a region thereof, which is an identifying region unique to calcitonin receptor; and

c) detecting binding of said antibody to said bacterial cells as an indication that said bacterial cells express a protein similar to calcitonin receptor.

32. A method of identifying a calcitonin receptor in a cell from a tissue, said method comprising

a) growing bacterial cells that express RNA specific for said tissue on a solid support;

b) hybridizing to said bacteria a labeled probe comprising a DNA sequence encoding calcitonin receptor or a portion thereof of greater than or equal to 30 base pairs, which contains an identifying region unique to calcitonin receptor; and

c) detecting hybridization of said probe to said bacterial cells as an indication that said bacterial cells express an RNA molecule specific for said calcitonin receptor.

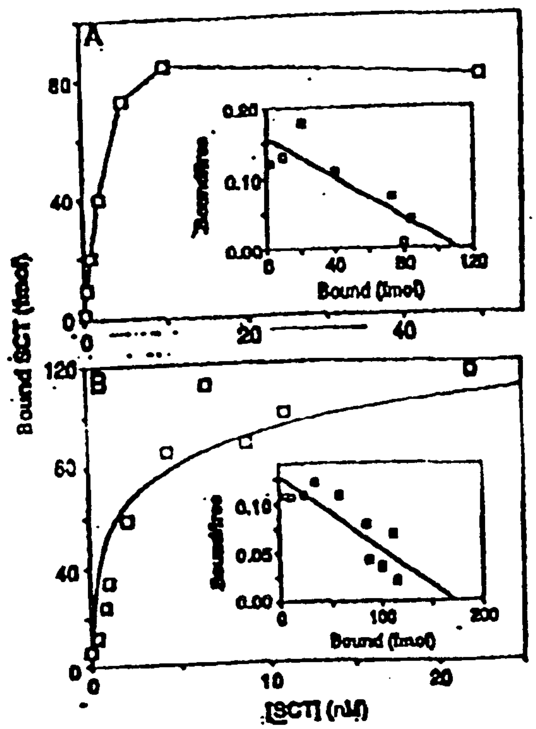
A
80
40
0
0
20
40
0.20
0.10
0.00
0
40
80
120
Bound/free
Bound (fmol)
Bound SCT (fmol)
120
B
80
40
0
0
10
20
0.10
0.05
0.00
0
100
200
Bound/free
Bound (fmol)
[SCT] (nM)

Figure 1

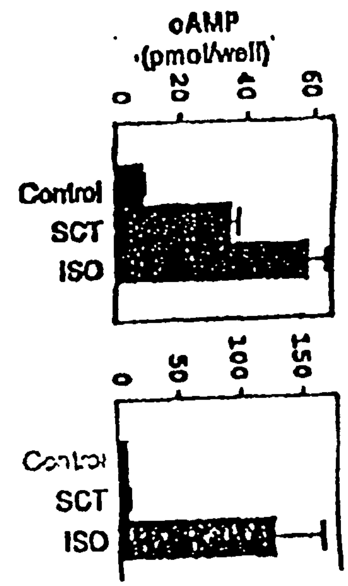
cAMP
(pmol/well)
0
20
40
60
Control
SCT
ISO
0
50
100
150
Control
SCT
ISO

Figure 2

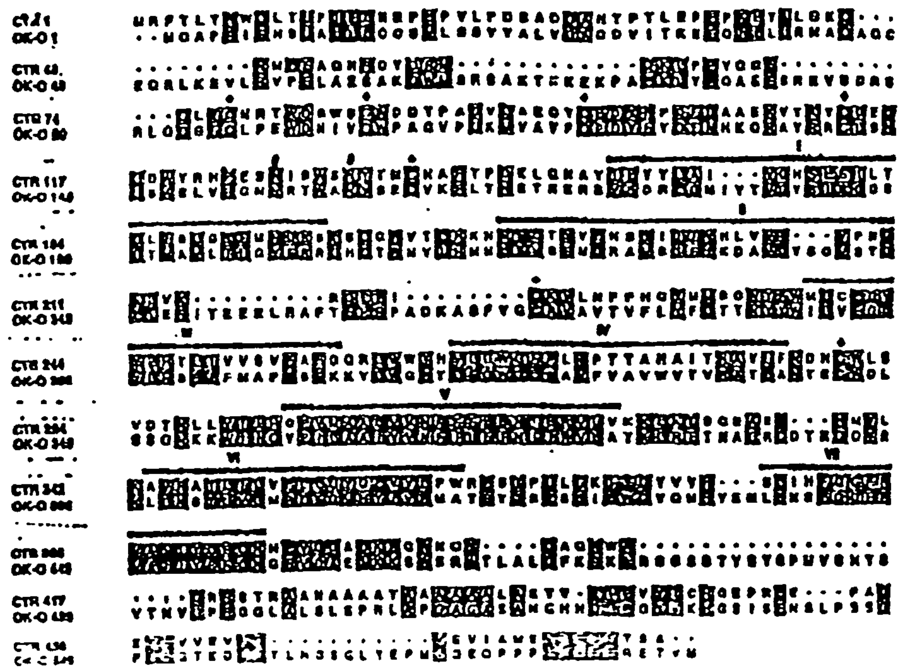

Figure 3

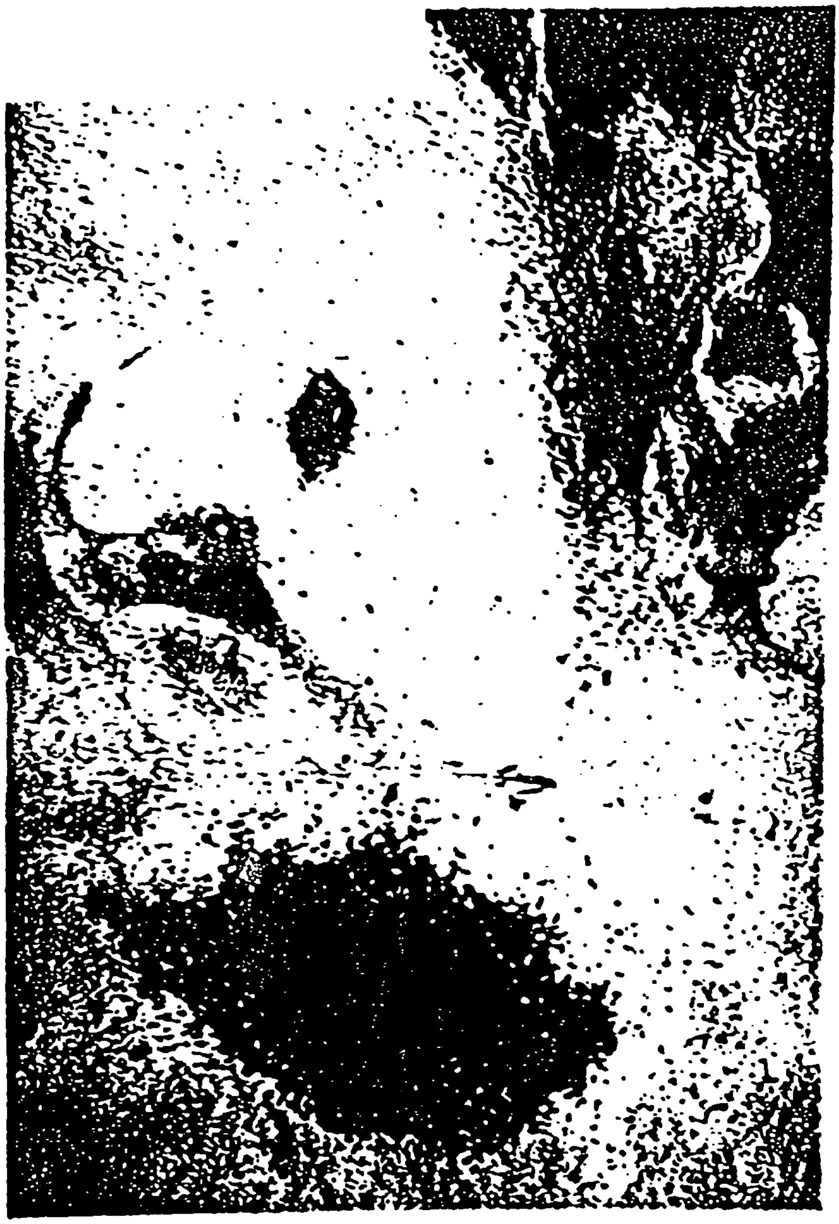

Figure 4A

Figure 4B

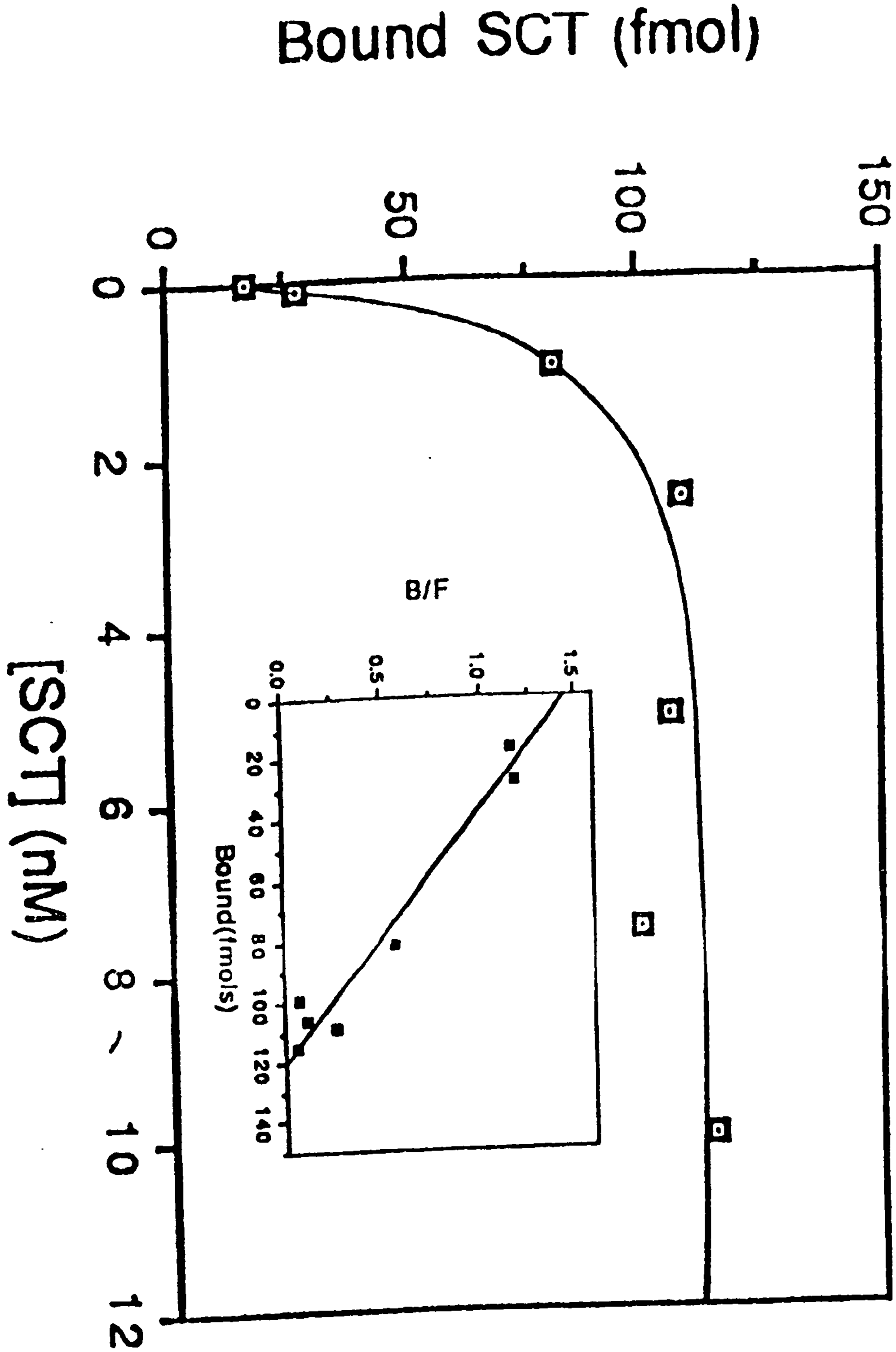
Bound SCT (fmol)
0
50
100
150
[SCT] (nM)
0
2
4
6
8
10
12
B/F
0.0
0.5
1.0
1.5
Bound(fmols)
0
20
40
60
80
100
120
140

Figure 5A

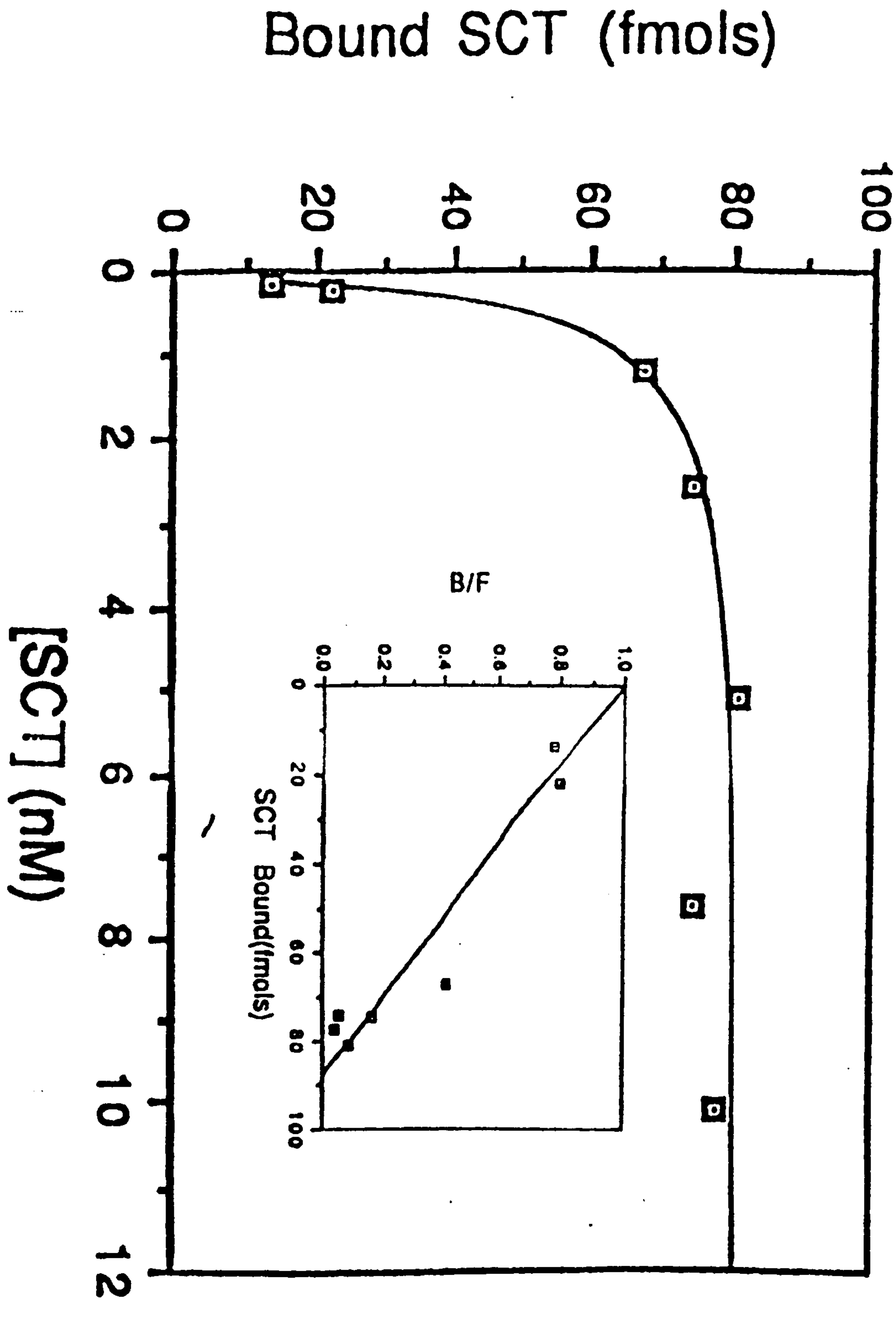
Bound SCT (fmols)
0
20
40
60
80
100
0
2
4
6
8
10
12
[SCT] (nM)
B/F
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
SCT Bound(fmols)

Figure 5B

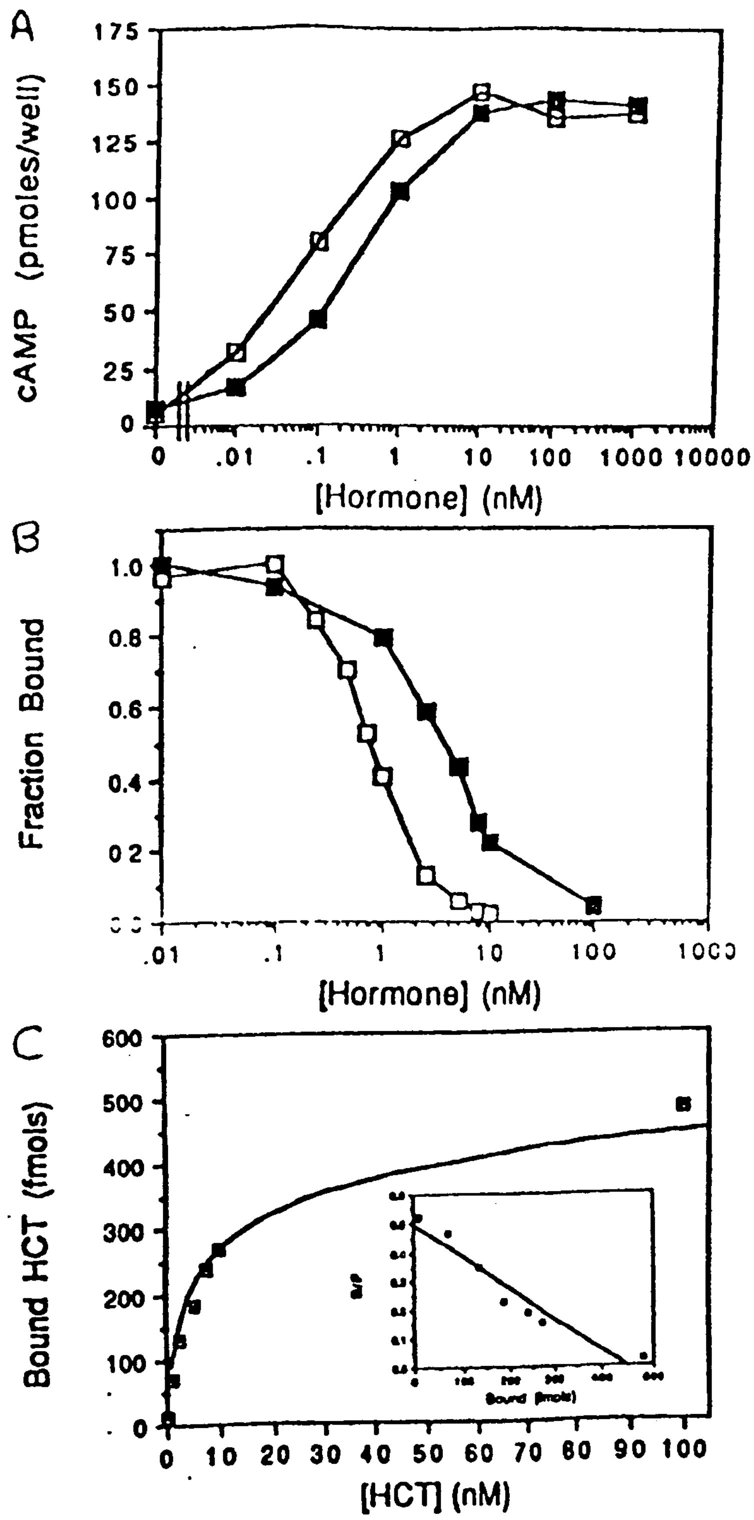
A
175
150
125
100
75
50
25
0
cAMP (pmoles/well)
0
.01
.1
1
10
100
1000
10000
[Hormone] (nM)
B
1.0
0.8
0.6
0.4
0 2
Fraction Bound
.01
.1
1
10
100
1000
[Hormone] (nM)
C
600
500
400
300
200
100
0
Bound HCT (fmols)
0
10
20
30
40
50
60
70
80
90
100
[HCT] (nM)

Figure 6

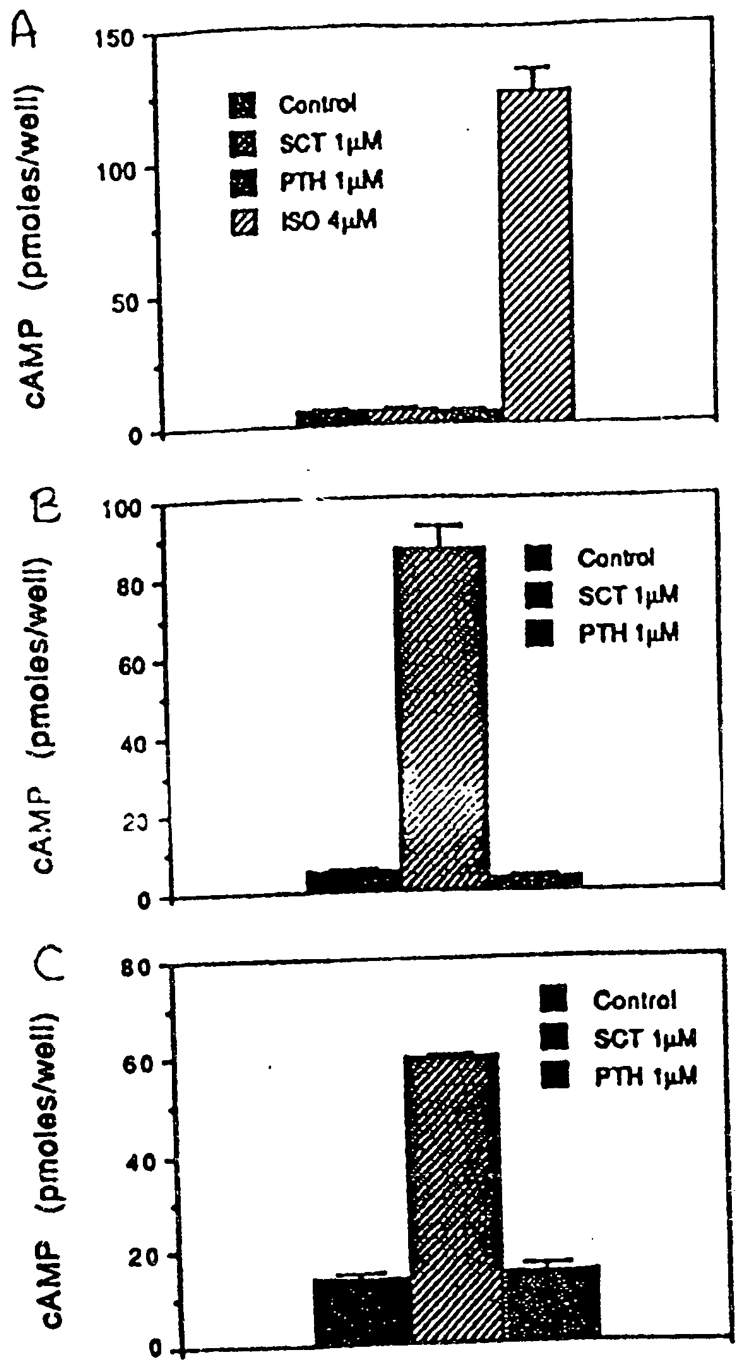
A
B
C
cAMP (pmoles/well)
150
100
50
0
80
60
40
20
Control
SCT 1μM
PTH 1μM
ISO 4μM

Figure 7

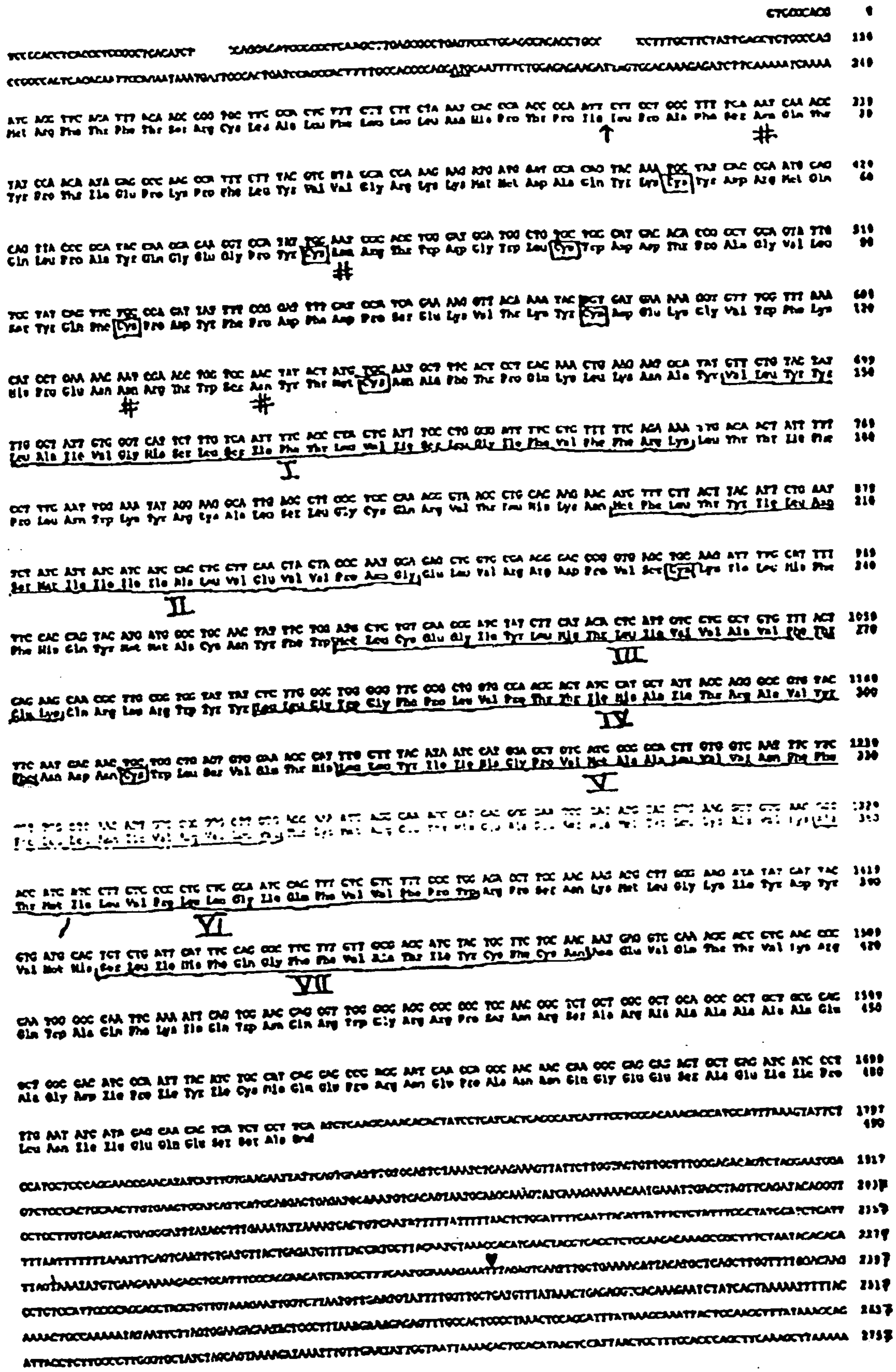

Figure 8

[illegible] 2877
[illegible] 2997
[illegible] 3117
[illegible] 3237
[illegible] 3357
[illegible] 3477
[illegible] 3597
CATCTTTT... 3605

Figure 8 (continued)

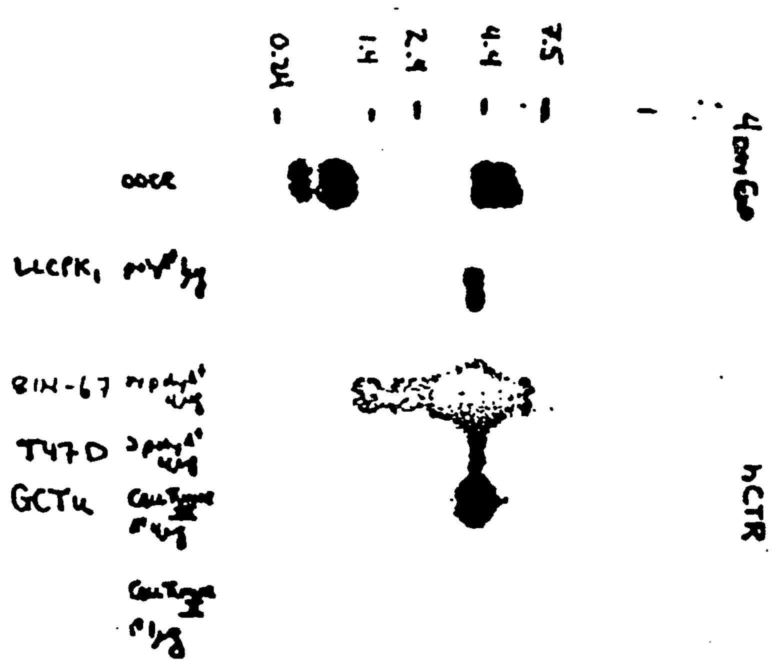

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09686

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 13/00; C12Q 1/02
US CL :435/7.72, 29; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.72, 29; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, EMBASE, MEDLINE, CA, APS. Search terms: mammalian calcitonin receptor, hypocalcemic factor.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Science Vol. 254, issued 15 November 1991, Lin et al, " Expression Cloning of an Adenylate-cyclase-coupled Calcitonin Receptor", pages 1022-1024, entire document.	1-16
A	Journal of Biochemistry, Vol. 250, issued 1922, Nicholson et al,"Human placental calcitonin receptors", pages 877-882, see 878 (first paragraph of first column).	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 05 January 1993	Date of mailing of the international search report
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PAUL B. TRAN, PH.D.
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-2127

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09686

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any addtional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention frist mentioned in the claims; it is covered by claims Nos.:
1-16 (telephone practice)

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-16, drawn to calcitonin receptors and methods of testing a compound capable of binding thereto, classified in classes 530/350, 435/7.72, and 435/29.
II. Claims 30 and 31, drawn to an antibody to calcitonin receptor and method of identifying a calcitonin receptor by immunoassay, classified in classes 530/387.1 and 435/7.2.
III. Claims 17-29 and 32, drawn to recombinant DNA encoding calcitonin receptors, vectors and hosts therefor, expression thereof, and method of detecting RNA encoding said receptor in a cell, classified in classes 435/320.1, 435/240.2, 435/69.1, and 435/6.